A THESIS FOR THE DEGREE OF

MASTER OF SCIENCE IN FOOD AND NUTRITION

Synthesis of Fucosylated Oligosaccharides Using Crude Enzyme Extracts from *Bifidobacterium longum* subsp. *longum* RD 47

Bifidobacterium longum subsp. *longum* RD 47 을 이용한 푸코실 올리고당 합성

February, 2014

Department of Food and Nutrition

The Graduate School

Seoul National University

Jeongeun Hwang

Abstract

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Human milk oligosaccharides (HMOs) are mostly fucosylated at their non-reducing termini. It has been proposed that HMOs function as a prebiotic for bifidobacteria. Through the screening procedure, crude enzyme extracts from *Bifidobacterium longum* subsp. *longum* RD 47 were newly found to produce fucosylated oligosaccharides. The synthesized oligosaccharides were further purified from reaction mixture and identified to be as fucosylated galacto-oligosaccharides and galacto-oligosaccharides by thin layer chromatography. The analysis of fucosylated oligosaccharides by mass spectrometry showed that degree of polymerization ranged from 2 to 7. To improve the yield of the production of fucosylated oligosaccharides, the effect of the medium composition on the production of crude enzyme extracts from *B. longum* RD 47 was evaluated and the oligosaccharides synthesis reaction conditions were

optimized. The addition of raffinose in culture medium resulted in increased galactosidase activities in crude enzyme extracts from *B. longum* RD 47. The synthesized fucosylated oligosaccharides may provide a new constituent for the HMOs and novel prebiotic food ingredients.

Keywords: L-fucose, oligosaccharides, *Bifidobacterium*, transglycosylation,

Student Number: 2012-21499

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List of abbreviations

DP: Degree of polymerization,

DW: Distilled water,

EDTA: Ethylenediaminetetraacetic acid

Fuc: L-Fucose

FUS: Fucosylated oligosaccharides

Gal: D-Galactose

Glu: D-Glucose

GOS: Galacto- oligosaccharides

HMO: Human milk oligosaccharide

Lac: D-Lactose

*p*NP: para-Nitrophenol

RD 47: *Bifidobacterium longum* subsp. *longum* RD 47

Raf: D-Raffinose

TLC: Thin layer chromatography

1. Introduction

1.1 Human milk oligosaccharides

Human milk oligosaccharides (HMOs) benefit infant intestinal microorganisms and provide protection against pathogens. HMOs are fermented by the intestinal microflora; which selectively stimulates the growth and activity of intestinal microorganisms which benefit the health of the host [1]. On the other hand infant intestinal bacteria such as bifidobacteria have the ability to hydrolysis of HMOs [2, 3]. HMOs are also very useful in the development of new infection protector or antiviral compounds.

HMOs have a core structure consisting of a lactose unit. The most abundant oligosaccharides are fucosylated and the proportion of fucosylated ones is approximately 77% [4]. In comparison to the commercially available oligosaccharides, which lack L-fucose found in HMOs, do not provide specific resistance to pathogens [5, 6]. L-fucose is also involved in a variety of biological events, such as cell surface recognition and antigenicity for enhancing host immunity [7, 8]. It has been suggested that HMOs serve as soluble receptors that prevent pathogens from adhering to and invading into the epithelial lining organs, such as the gastrointestinal and respiratory tracts [5].

1.2 Synthesis of novel fucosylated oligosaccharides

Since the understanding of the biological functions of the HMOs has been increased, the need for the development of the practical synthetic procedures of the HMOs and their analogs has become attractive subjects [9, 10]. Nonetheless, with their structural and compositional complexity, HMOs currently cannot be industrially produced. There have been various attempts for the production of fucosylated oligosaccharides (FUS) by chemical glycosylation of mono- and disaccharides [11].

Chemical synthesis requires repetitive, multiple steps for each HMOs' molecule which decrease the yields and productivity [12]. Alternatively, biocatalytic strategy using enzymes or whole microbial cells may be more efficient for the production of FUS.

The enzymatic approach has utilized various glycosyltransferases and glycosidases [13, 14]. However, among the enzymatic processes for FUS production, use of glycosyltransferases may be less desirable for large-scale synthesis. Because multiple steps of the required enzymes and cofactors are needed and an expensive sugar nucleotide or a complex system for nucleotide recycling is required [15, 16].

From a practical viewpoint, the use of glycosidases for transglycosylation reactions has drawn more attention. Various studies on the production of galactosyl- oligosaccharides (GOS) residue using β-D-galactosidase with lactose as a galactose donor have been performed [17, 18]. Consequently, lactose with some trans-galactosidases could produce GOS with the following acceptors: D-mannose, L-fucose, D-fructose, D- sucrose, D- maltose, D-xylose, N-acetyl-glucosamine, N-acetyl-galactosamine and etc. [19]. However, there

have been scarce reports on the production of FUS using transgalactosylation reaction. Therefore L-fucose could be used as a substrate for the enzymatic synthesis of FUS that may provide new opportunities in the development of future prebiotics.

In the present study, we screened and selected a relatively strong producer of FUS among various lactic acid bacteria. Here, we represent the synthesis, purification and characterization of the synthesized FUS using a selected *Bifidobacterium.*

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma Aldrich (Sigma, St, Louis, MO, USA) except L-fucose (Jennewein Biotechnologie GmbH, Rheinbreitbach, Germany). All chemicals used in this study were of analytical grade. Bifidobacteria and lactobacilli strains, isolated from the faeces of adults and infants, were obtained from the Food Microbiology Laboratory Strain Collection at the Seoul National University (*Lactobacillus* spp. *Bifidobacterium* spp.) or from the Korean Collection for Type Culture.

2.2. Screening of galactosidase activity in crude cell extracts

2.2.1. Culture conditions

All bacteria were activated by two successive precultures in Man Rogosa Sharp (MRS) medium (Difco, Detroit, MI, USA) with 0.05% (w/v) cysteine-HCl (Sigma, St, Louis, MO, USA) at 37℃ for 18 h. The activated bacteria were inoculated in 8 mL MRS and BHI, both containing 0.05% (w/v) cysteine-HCl and various sugars. Strains were grown at 37℃ for 18 h under anaerobic conditions.

2.2.2. Preparation of crude cell extracts

Bacteria grown in the each medium at 37℃ for 18 h were centrifuged (16,000 × g, 5 min). Cells were harvested by centrifugation, washed twice in 50 mM sodium phosphate buffer (PB, pH 6.6) and the supernatant was discarded.

For preparation of crude enzyme extracts, washed cells were resuspended in 1 volume PB (pH 6.6) and disrupted with a sonicator (Sonicator 500, Q-Sonica , U.S.A.) in 1.0 s on /1.0 s off intervals for 5 min. Supernatants were used after centrifugation (16,000 x g for 12 min at 4℃).

2.2.3. Determination of hydrolytic activity in crude cell extracts

Relative enzyme activity was measured by using the para-nitrophenol (*p*NP) -D–α-/β-galacto-pyranosides as substrates. Enzyme solution (80 μl, 5 μl crude enzyme extracts in 75 μl PB) was added to 20 μl 5 mM *p*NP-D-galactoside in 50mM sodium phosphate buffer (PB, pH 6.6). The mixture was incubated at 37℃ for 10 min. The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. Activity was measured in microplates at 405 nm in a spectrophotometer. Specific activity (enzyme activity level relative to cell mass) was determined as units of galactosidase activities.

2.2.4. Determination of transglycosylation activity in crude cell extracts

Transglycosylation activities were assessed using crude enzyme extracts of *Bifidobacterium* spp. cultured in basal medium with 2 % (w/v) raffinose.

Lactose (400 mg) and fucose (400 mg) were mixed and finally 2 ml solution in PB (pH 6.6) was prepared for reaction. The enzyme extract (0.1 unit, 0.3 ml) was added to sugar solution. The mixture was incubated at 37℃. After 12-h incubation, the reaction was terminated by boiling for 10 min.

The carbohydrates in the reaction products were determined by thin-layer chromatography (TLC). The mixtures of transglycosylation products were spotted on a silica gel plate 60 (Merck, Darmstadt, Germany). The plate was developed with a 1-propanol: DW: ethyl-acetate (7:2:1) solvent solution, then visualized using a sulfuric acid-ethanol (1:9, v/v) solution and dried plate was in an oven (120℃) for 5 min. The compounds in the reaction mixtures were identified by comparing the retention factors (Rf) with those of standard sugars.

To further determine the pattern of oligosaccharides, products of the enzymatic reactions were analyzed by two-dimensional (2D) TLC to separate oligosaccharides with different degree of polymerization (DP). The first developing solvent was 1-propanol: DW: ethyl-acetate (7:2:1, v/v/v). After two ascents, the TLC plate was dried, rotated 90; then it was developed in a second solvent (ethyl-acetate: 1-propanol: DW: acetic acid 4:2:2:1, v/v/v/v). Visualization was conducted as previously mentioned.

2.3. Characterization of crude cell extracts from a selected bacterium

2.3.1. Changes of galactosidase production in crude cell extracts from RD 47 during cultivation

The activated bacteria were cultured in modified MRS containing 0.05% (w/v) cysteine-HCl and 2% raffinose. After lag phase, each of cells grown in 8, 12, 15, 18, 24 and 30 h was harvested by centrifugation (16,000 x g, 5 min) and pellets were stored in -80℃ until used. The crude enzyme extracts of different cultivation time were collected after sonication as mentioned above.

Relative enzyme activity and transglycosylation activities were determined using the same methods as described above.

2.3.2. Effect of enzyme extraction methods on galactosidase activity in crude cell extracts from RD 47

To find a better enzyme extraction method, different chemical and enzymatical methods were used to disrupt RD 47 cells. The cells were resuspended in detergents such as 0.12% (w/v) cetridium bromide (CTAB) or 0.25% (w/v) Triton X-100 in 1ml PB (pH 6.6), and centrifuged at 16,000 x g for 3 min. The supernatant was used for the enzyme assay. Cell suspensions were shaken in the presence of 1.5% (w/v) lysozyme for 5 min and sonicated for 2 min. After centrifugation at 16,000 x g for 3 min, the supernatant and pellet were used for the enzyme assay. Hence, the effect of these methods on enzyme activity was compared with the sonication method.

2.3.3. Substrates specificity of the crude cell extracts from RD 47

The hydrolytic activity of RD 47 towards substrates with different glucosidic linkages was measured with 5 mM *p*NP-D-glucoside, *p*NP-D-galactoside, *p*NP-L-fucoside suspended in 50 mM PB. Substrates specificity toward various sugars was assessed with 100 mM of various sugars in 50 mM PB (pH 6.3) and 0.1 unit enzyme at 37℃ for 24 h. The reaction was stopped by boiling for 10 min and products were analyzed by same method mentioned above.

2.3.4. Effect of various metals ions and enzyme inhibitors on galactosidase activity in crude enzyme extracts from RD 47

Crude enzyme extracts from RD 47 with 0.1 units of enzyme activity were mixed with 1 mM metal ions, glycerol, EDTA and protease inhibitor cocktails (P8215 and P8426, Sigma). Solutions were incubated in water baths at 37 and 50℃ for 1 h, 1 d and 4 d. Assays were performed as section 2.2.3.

2.3.5. Time and temperature dependent of crude enzyme extracts from RD 47

The crude enzyme extracts from RD 47 and reaction mixtures were withdrawn at 1-day intervals. The changes of galactosidase activity were determined during storage at 37, 50 and 60℃, respectively. Assays were performed as section 2.2.3 and 2.2.4.

2.4. Optimization of the synthesized oligosaccharides produced by RD 47

2.4.1. Effect of carbon sources in culture medium on galactosidase activity in crude enzyme extracts from RD 47

The basal medium was prepared according to the composition of MRS excluding dextrose. After sterilization, a filter sterilized sugar (20 g · L^{-1}) such as glucose, galactose, lactose and raffinose was added to the basal medium as the sole carbohydrate source.

2.4.2. Concentration of crude enzyme extracts from RD 47

Crude enzyme extracts from RD 47 were concentrated by two steps. Before sonication, the cell pellets grown in 50 ml medium were suspended in 2 ml of PB. And supernatant from sonication was concentrated using centricon centrifugal filter devices (Amicon, Millipore, USA) with 100,000 NMWL (2,000 x g, 10 min). The unit of crude enzyme extracts was adjusted to the adequate amounts by elution in PB.

2.4.3. Effect of pH and temperature on galactosidase activity crude enzyme extracts from RD 47

For the measurement of the temperature and pH profiles, the crude enzyme extracts from RD 47 in 50 mM PB were used. Before sonication, whole cells were resuspended in different pH PB buffer then crude enzymes extracts from

RD 47 were collected, respectively. *p*NP assay was also performed to determine the optimal temperature at the range of 35-51℃ and pH 5.5–7.5.

2.4.4. Effect of additional substances in reaction mixtures on galactosidase activity in crude enzyme extracts from RD 47

In order to investigate the influences of additional substances on the formation of transglycosylation products, several substances (such as poly ethylene glycol, dimethyl sulfoxide, glycerol, sorbitol and Tween 20) were added to lower water activity. An aliquot of 10 μL of crude enzyme extracts from RD 47 with 0.1 units of enzyme activity were applied to 20 μL of solutions of 20% (w/w) lactose and 20% (w/w) fucose with 10 μL of 40 % (w/v) substances for a final concentration of 10% (w/w) each sugars and 10% (w/w) substances. Assays were performed as section 2.2.4.

2.4.5. Effect of *Saccharomyces cerevisiae* on galactosidase activity in crude enzyme extracts from RD 47

Glucose, a noncompetitive inhibitor of β-galactosidase activity, was removed using *S. cerevisiae* KCTC 7919 in reaction mixtures to enhance transglycosylation activity of crude enzyme extracts from RD 47. All samples, along with a control solution of 1 mL crude enzyme extracts with 0.1 units of β-galactosidase activity in 3 mL of 20% (w/w) lactose and fucose, were aerobically incubated in shaking test tubes at 30℃. The reaction was stopped by boiling at 100℃ for 5 min. After enzyme inactivation, all samples were

centrifuged for 5 min at 16,000 x g to remove *S. cerevisiae* from reaction mixtures. Assays were performed as described in section 2.2.4.

2.5. Mass analysis of the synthesized oligosaccharides

2.5.1. Mass analysis by MALDI-TOF/MS and LC-ESI/MS

Purification was performed following the method of Morales and others [20]. Briefly, a total of 2.0 mL of reaction mixture, containing 0.8 g of carbohydrates, was dissolved in 10 mL of a 1% aqueous solution of ethanol and stirred for 30 min with 500 mg of nonporous graphitized carbon solid phase extraction cartridges (Superclean ENVI-Carb, Supleco, USA), to remove mono- and disaccharides. This mixture was filtered through syringe filter, and activated charcoal was washed with 5 mL of water. The oligosaccharides adsorbed onto the activated charcoal were extracted by stirring for 30 min with 10 mL of ethanol/water solution (1:1, v/v) and then filtered. The ethanol/water solution was evaporated under vacuum at 30℃. The sample was dissolved in 5 mL of deionized water and filtered through 0.22 μm filters (Whatman no. 1, Millipore Corp., Bedford, MA).

After purification, the reaction mixtures were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on Voyager-DETM STR Biospectrometry Workstation mass spectrometer (Applied Biosystems, Foster City, CA) at NCIRF of Seoul National University. Mass spectra were obtained over the m/z range 150-2000.

Also mass analysis using LC/MS system was performed at NICEM of Seoul National University. LC-ESI/MS analysis performed in the positive ionization modes exhibited saccharides in the m/z 150 to 2000.

2.6. Separation of crude oligosaccharides mixtures

2.6.1. Size exclusion chromatography by bio gel P 2 column

Monosaccharides present in oligosaccharides crude extracts were removed by size exclusion chromatography (SEC) on Bio gel P-2 column (100 x 40 mm, i.d, GE Healthcare, USA) and Ä KTA prime system (GE Healthcare, USA). The oligosaccharides mixtures (2 g resolved in PB) were loaded onto a column of Bio gel P2, respectively. The column was eluted by sterilized water at a flow rate of 0.6 mL/min. Ninety fractions (12 ml) were collected and concentrated by a speed vacuum concentrator (ScanSpeed 40, LaboGene, Denmark). Concentrated fractions were analyzed by TLC using the same mobile phase as described above.

2.6.2. Preparative thin layer chromatography

Preparation of oligosaccharides and analysis of its component was performed following the method of Suh [21]. Briefly, a mixture of oligosaccharides was spotted on preparative silica gel glass plates (Merck, USA). The glass plate was developed using a solvent of 1- propanol: DW: ethyl-acetate (7:1:2, v/v/v), and then a vertical side of the end was removed for visualization. The cut strip was compared with the undeveloped portion of the silica gel plates, and silica gel

containing each designated component was separately gathered. In order to obtain carbohydrates each silica gel fraction was suspended in DW and centrifuged (5,000 x g for 10 min at 4℃). Each component was identified by using TLC then evaporated on speed vacuum system and stored -20℃.

Fractionated oligosaccharides were digested by the enzyme extracts from RD 47 and other bifidobacteria to verify whether the novel oligosaccharides contained L-fucose or D-galactose.

3. Results

3.1. Screening of galactosidase activity in crude cell extracts

Sixty strains of lactic acid bacteria were screened for the enzymatic activity toward not only hydrolysis but also transglycosylation. Relative enzyme activities of the crude enzyme extracts from 7 selected bifidobacteria grown in the presence of lactose and raffinose was measured. Crude enzyme extracts of all strains tested hydrolyzed *p*NP- α/β- galactoside (Fig. 1). The addition of raffinose in medium resulted in the elevated α-galactosidase activity.

Among the tested bifidobacteria, *B. longum* RD 47 showed considerable transglycosylation ability to several sugars. When lactose was used as a substrate, oligosaccharides were formed with various DP (Fig. 2). Finally, strain RD 47 with the strongest ability was chosen for further studies.

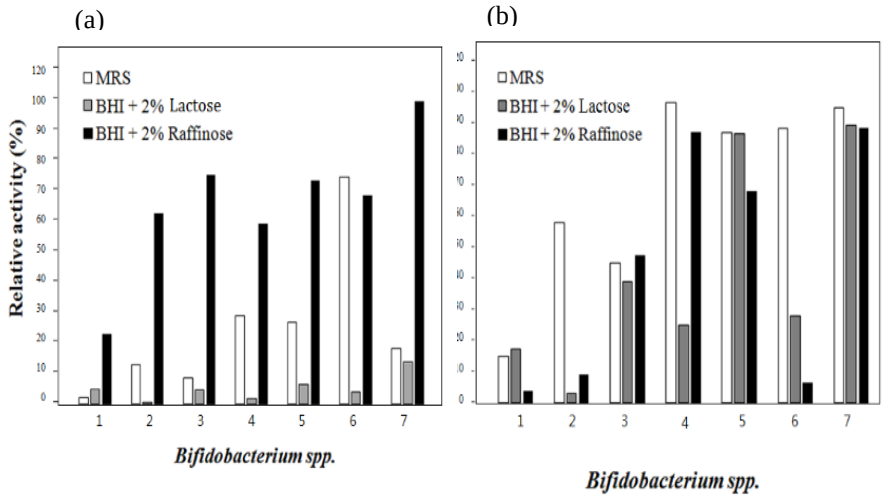

Figure 1. Screening of α-(a) and β- (b) galactosidase activity of crude enzyme extracts from 7 selected bifidobacteria. (1: *B. longum* SJ 32, 2: *B. longum* RD 81, 3: *B. longum* RD 01, 4: *B.breve* KCTC 3419, 5: *B. adolescentis* Int 57 6*: B. longum* RD 47)

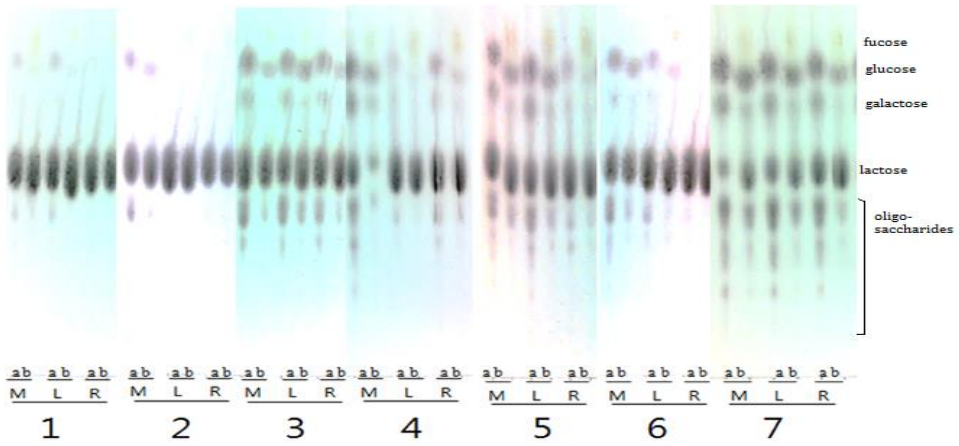

Figure 2. Screening of transglycosylation ability of crude enzyme extracts from 7 selected bifidobacteria. (1: *B. longum* SJ 32, 2: *B. longum* RD 81, 3: *B. longum* RD 01, 4*: B.breve* KCTC 3419, 5: *B. adolescentis* Int 57 6: *B. longum* RD 47; oligosaccharides were synthesized from lactose (a) and lactose and fucose (b) as substrates; M: cell cultured in MRS, L: cell cultured in modified MRS with lactose, R: cell cultured in modified MRS with raffinose)

3.2. Determination of crude cell extracts from RD 47

3.3.1. Changes in crude galactosidases production in crude enzyme extracts from RD 47 during cultivation

The results in Fig. 3 show that both α and β galactosidases production during cultivation were optimal at 18 h. It was suggested that maximal enzyme production was related to cell growth. Since the amount of transglycosylation products was highest at 18 h, the hydrolytic and transglycosylation ability proceeded with similar ratio (Fig. 4).

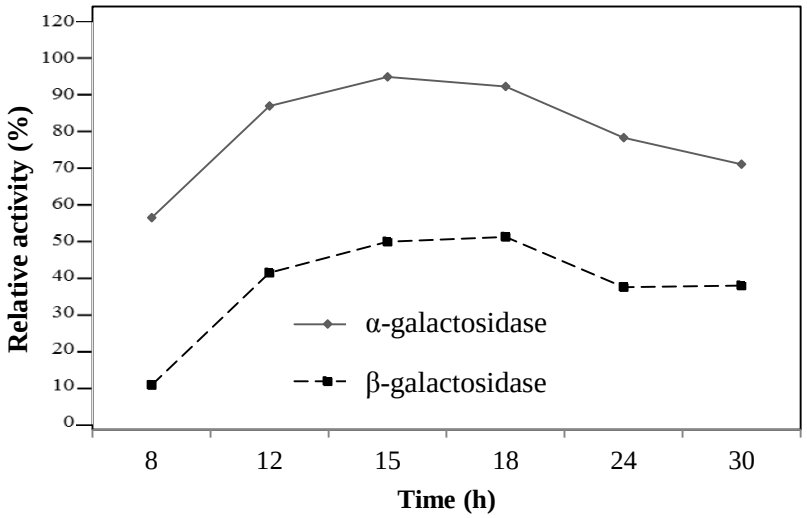

Figure 3. Relative activity of crude enzyme extracts from RD 47 during cultivation.

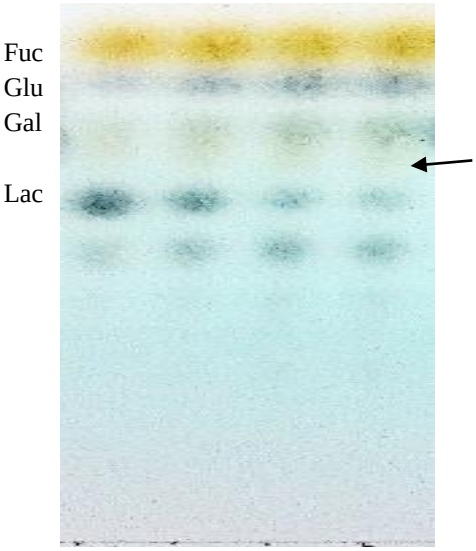

Figure 4. Transglycosylation activity of crude enzyme extracts from RD 47 during cultivation. Analysis of reaction mixture with lactose and fucose was performed by TLC.

3.2.2. Comparison of different enzyme extraction methods on galactosidase activity in crude enzyme extracts from RD 47

The results indicated that the specific activity of β-galactosidase of crude enzyme extracts from RD 47 was not considerably different from the cell disintegration method (Fig. 5 and 6). Lysozyme, which hydrolyze the bacteria glycan, was also used for cell disruption. However crude enzyme extracts from RD 47 were precipitated when lysozyme was added to the cell suspension or supernatants of disrupted cell suspension (Fig. 7 and 8).

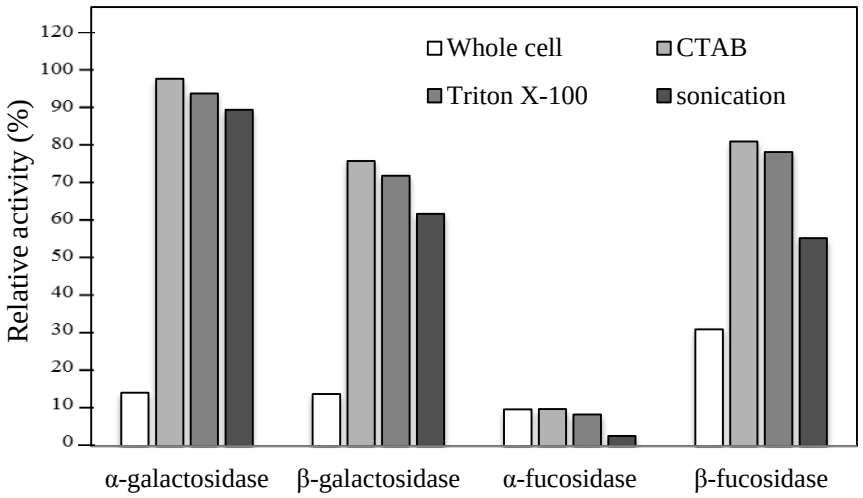

Figure 5. Effect of different enzyme extraction methods on relative activity of crude enzyme extracts from RD 47.

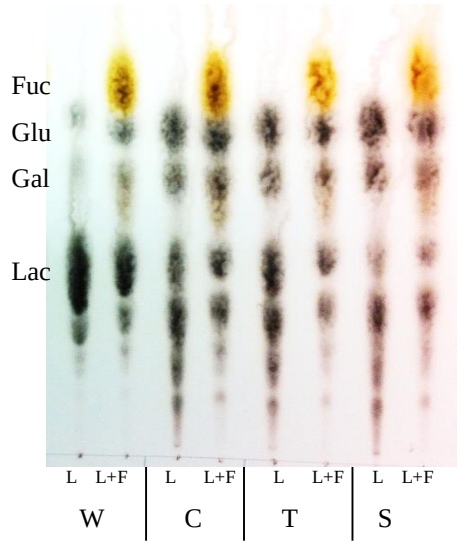

Figure 6. Effect of different enzyme extraction methods on transglycosylation activity of the crude enzyme extracts from RD 47. W: whole cell suspension, C: supernatant of cell suspension treated with CTAB, T: supernatant of cell suspension treated with Triton X-100, S: supernatant from sonication. Analysis of reaction mixture with lactose (L) and lactose and fucose (L+F) was performed by TLC.

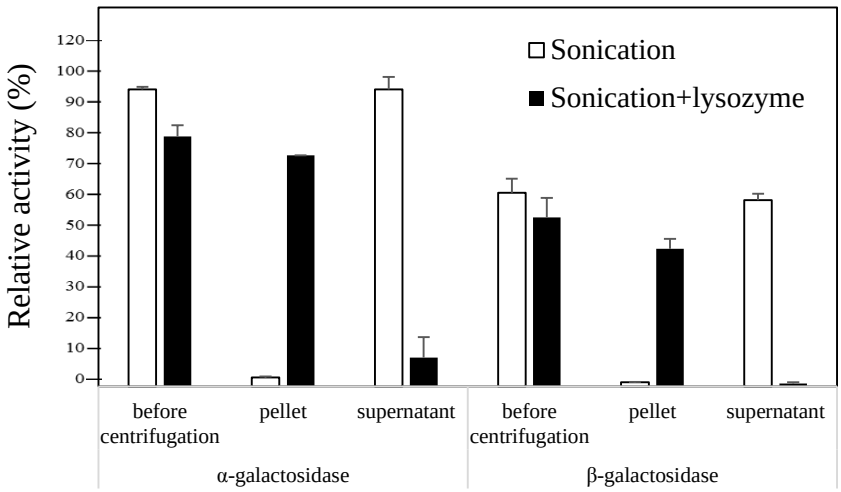

Figure 7. Relative enzyme activity of lysozyme-treated cell suspension.

Figure 8. Transglycosylation activity of lysozyme-treated cell suspension. 1: RD 47 cell suspension before centrifugation, 2: pellets, 3: supernatants. Analysis of reaction mixture with lactose and fucose was performed by TLC.

3.2.3. Substrate specificities of crude cell extracts from RD 47

The data of substrate specificities were obtained from the TLC analysis (Fig. 9, Table 1). Crude enzyme extracts from RD 47 hydrolyzed *p*NP-α/β-glucoside, *p*NP-α/β-galactoside, *p*NP-β-fucoside, but not *p*NP-α-fucoside (Table 1). Among them, the crude enzyme extracts from RD 47 rapidly hydrolyzed the *p*NP-α/β-galactoside.

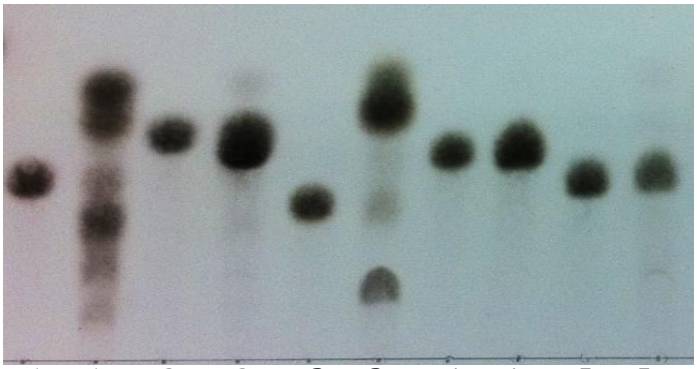

Figure 9. Transglycosylation profile of crude enzyme extracts from RD 47. 1: lactose, 2: maltose, 3: raffinose, 4: melizitose, 5: maltotriose, +: mixture with sugar and crude enzyme extracts from RD47

Table 1. Substrate specificity of crude enzyme extracts from *B.longum* RD 47

Substrate	Hydrolysis	Transglycosylation
*p*NP-α-glucoside	+	ND
Trehalose (Glu α-1,1 glu)	+	+
Maltose (Glu α-1,4 glu)	+	+
Isomaltose (Glu α-1,6 glu)	+	+
Melizitose (Glu α-1,6 *p*fru)	+	+
*p*NP-β-glucoside	+	ND
Cellobiose (Glu β 1,2- glu)	+	+
*p*NP-α-galactoside	+	ND
Melibiose (Gal α-1,6 Glu)	+	+
Sucrose (Glu α-1,2-β *p*fru)	+	+
Raffinose (Gal α-1,6 Glu)	+	+
Stachyose (Gal α-1,6 Glu)	+	+
*p*NP-β-galactoside	+	ND
Lactose (Gal β-1,6 Glu)	+	+
*p*NP-α-fucoside	-	ND
3'fucosyl-lactose (Fuc α-1,3 Lac)	-	ND
*p*NP-β-fucoside	+	ND

ND: not determined, +: detected, -: not detected. Relative enzyme activity was performed by using *p*NP as substrates. Substrate specificity of crude enzyme extracts from *B. longum* RD 47 was determined by TLC.

3.2.4. Effect of various metal ions and enzyme inhibitors on galactosidase activity in crude enzyme extracts from RD 47

The results (Fig. 10) show that some metal ions such as magnesium, manganese and calcium elevated galactosidase activity whereas addition of poly ethylene glycol (PEG) and glycerol decreased the activity. Presence of 0.5 mM sodium EDTA influenced the β- galactosidase activity of crude enzyme extracts from RD 47 toward transgalactosylation at incubation temperatures of 50℃ (Fig. 10 and 11). Addition of EDTA decreased the β-galactosadase activity. However at high temperatures EDTA stabilized the enzyme activity. The decreasing degree of the activities at high temperatures declined by the treatment 1 mM EDTA (Fig. 12).

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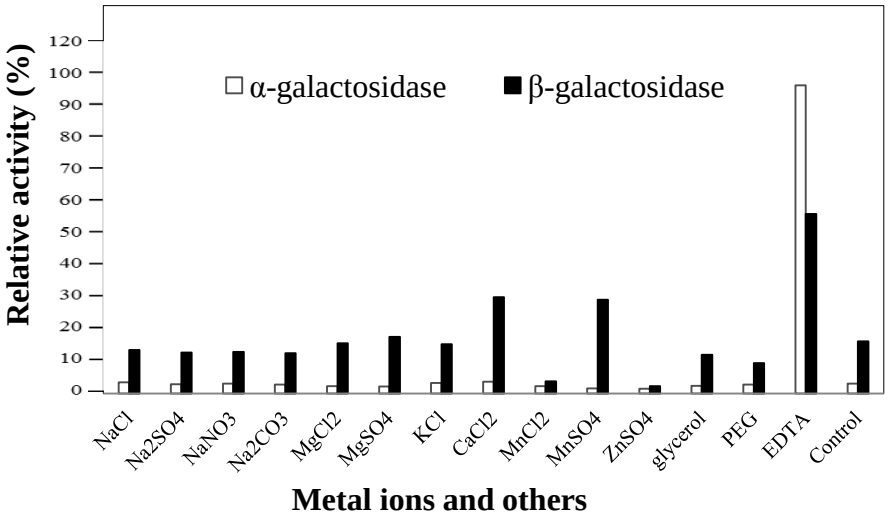

Figure 10. Relative activity of crude enzyme extracts from RD47 in the presence of various metal ions and others at 50℃.

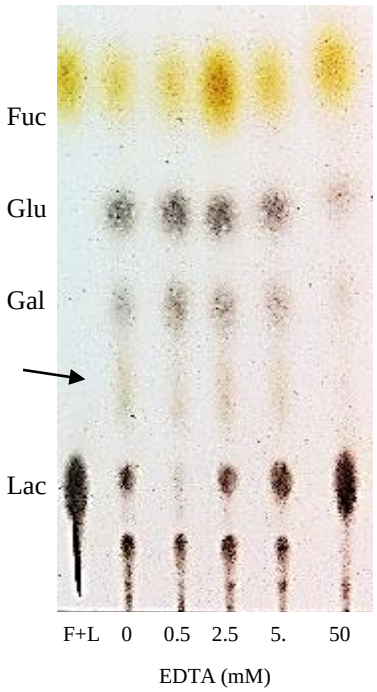

Figure 11. Effect of EDTA concentration on transglycosylation activity of crude enzyme extracts from RD47 at 50℃. Analysis of reaction mixture with lactose and fucose was performed by TLC.

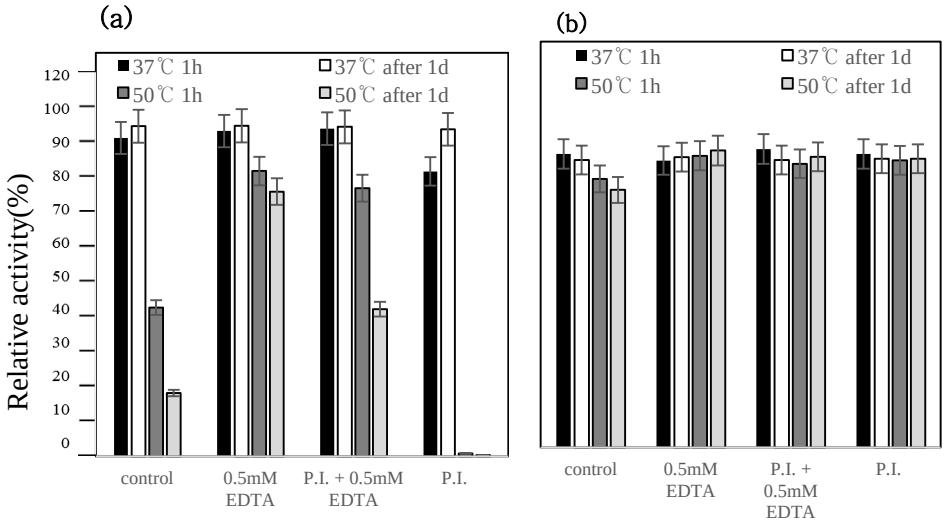

Figure 12. Relative activity of crude enzyme extracts from RD 47 in the presence of various protease inhibitor cocktails. (a): α- galactosidase, (b): β-galactosidase, P.I.+ 0.5 mM EDTA : Protease inhibitor cocktail (P8215) P.I.: Protease inhibitor cocktail (P 8426)

3.2.5. Time and temperature dependent of galactosidase activity in crude enzyme extracts from RD 47

Transglycosylation activities were observed from the initial reaction period (Fig. 13). After lactose was fully hydrolyzed to glucose and galactose, synthesized saccharides were also diminished over the reaction time. The maximum amount of the oligosaccharides was obtained by terminating the reaction before all lactose was consumed. At 37℃ the degree of β- galactosidase activity rapidly decreased, whereas α-galactosidase activity only mildly decreased (Fig. 14).

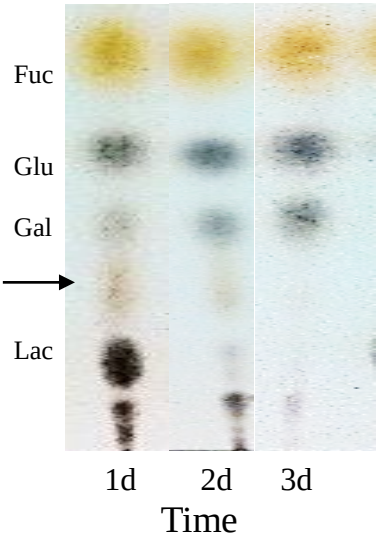

Figure 13. Effect of the reaction time on the formation of transglycosylation products. Analysis of reaction mixture with lactose and fucose was performed by TLC.

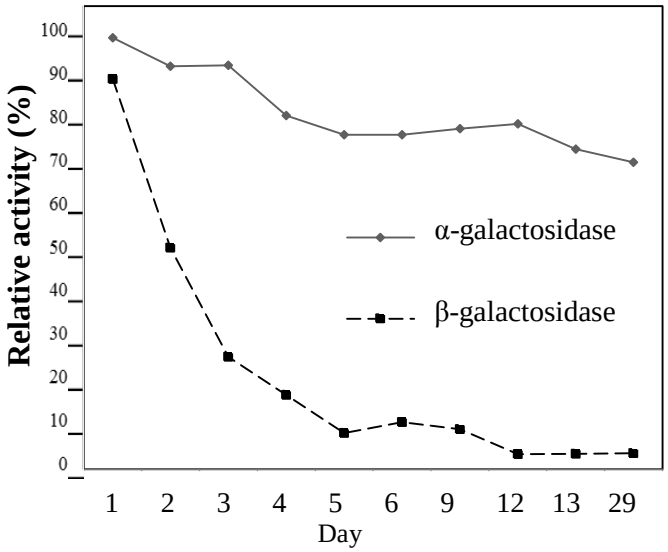

Figure 14. Relative activity of crude enzyme extracts from RD 47 during storage at 37℃.

3.3. Optimization of transglycosylation activity of galactosidase in crude enzyme extracts from RD 47

3.3.1. Effect of carbon sources in culture medium on galactosidase activity in crude enzyme extracts from RD 47

The activities of α/β- galactosidase of crude enzyme extracts from RD 47 were optimal when RD 47 was grown in modified MRS medium containing 2% (w/v) raffinose. The results showed that RD 47 presented the highest levels of α -galactosidase on raffinose, whereas the crude β-galactosidase activity was not significantly changed (Fig. 15). The β -galactosidase in RD 47 showed the highest levels of activity compared to the cell mass in the presence of raffinose as a substitute of glucose at 18 h of cultivation at 37℃. Also, the high production of FUS was observed from RD 47 grown in the same culture medium.

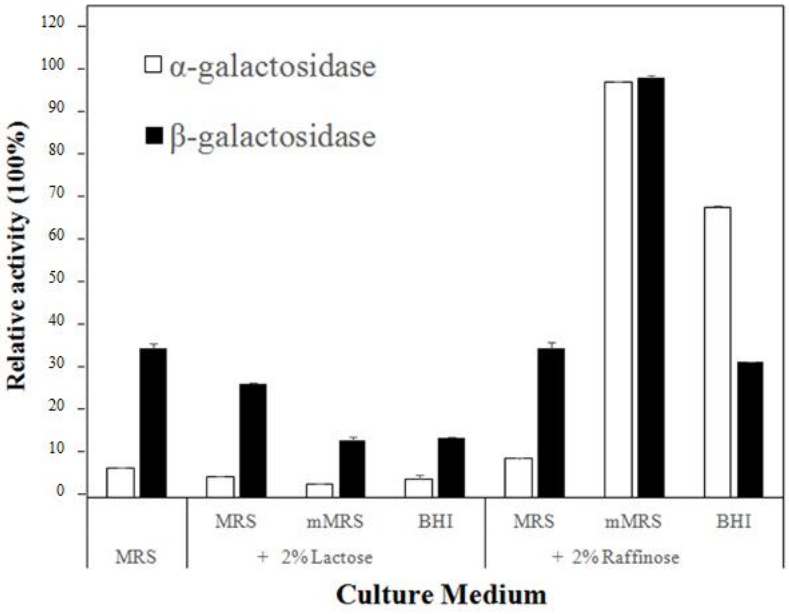

Figure 15. Relative enzyme activity of crude enzyme extracts from RD 47 grown in various medium.

3.3.2. Effect of pH and temperature on galactosidase activity in crude enzyme extracts from RD 47

The temperature and pH dependence of activities were shown in Fig. 16. The results of pH and temperature were presented with the highest value of each set, being assigned the activity value of 100%. Each of the experiments were carried out at least three times. The optimal temperature and pH were 42℃ at pH 6.3 or 6.6 for α-galactosidase (Fig. 16 A). Hydrolysis activity at optimal level for β-galactosidase was observed in sodium phosphate buffer pH 6.3 at 37℃ or pH 6.0 at 35℃ (Fig. 16 B). The results showed that the optimal temperature of α- galactosidase was slightly higher than that of β –galactosidase.

The optimal temperature of transglycosylation production is similar to that of the optimal exhibiting its hydrolytic activity, which was determined to be at 37℃ using lactose as a substrate (Fig. 17). The reaction was maximal when crude enzyme from RD 47 was incubated with the substrates in sodium phosphate buffer (pH 6.6) at 37℃ for 15 h.

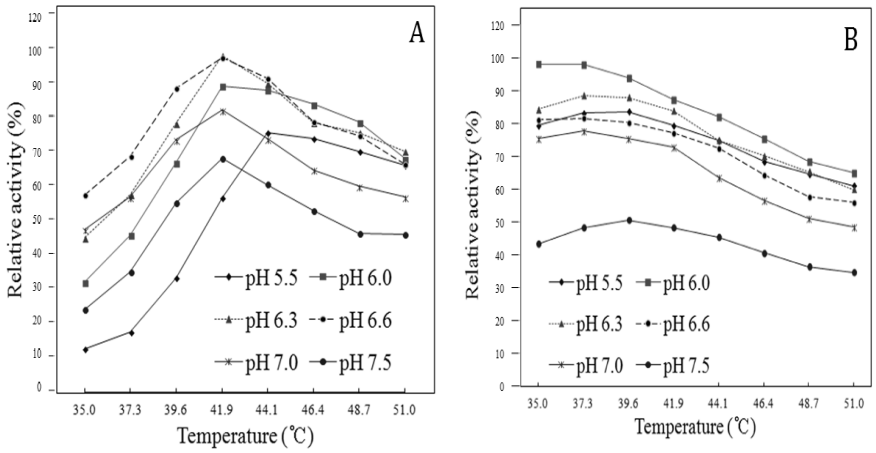

Figure 16. Effect of pH and temperature on relative activity of crude enzyme extracts from RD 47. (A): α-galactosidase, (B): β- galactosidase.

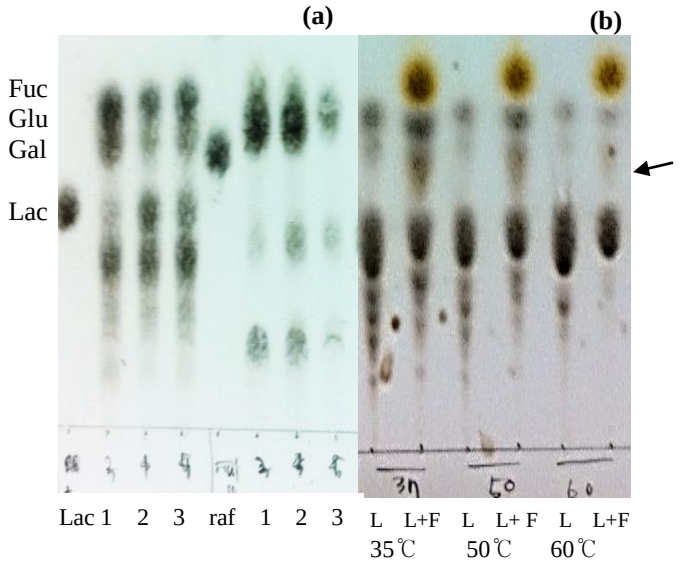

Figure 17. Effect of pH (a) and temperature (b) on transglycosylation activity of crude enzyme extracts from RD 47. (a) 1: pH 6.3, 2: pH 6.8, 3: pH 7.1, (b): Analysis of reaction mixture with lactose (L) and lactose and fucose at various temperature was performed by TLC.

3.3.3. Effect of additional substances in reaction mixtures on galactosidase activity in crude enzyme extracts from RD 47

Transglycosylation activity was enhanced at higher lactose concentrations, corresponding to reduced water activities. By removing the available water, there is a greater opportunity for sugars to serve as acceptor molecules following lactose hydrolysis. In this study the additional substances (such as poly ethylene glycol, dimethyl sulfoxide, glycerol, sorbitol and Tween 20) were added to lower water activity (Fig. 18). Other substance did not show considerable effects on oligosaccharides production whereas glycerol inhibited the transglycosylation activity. Remarkably, the addition of sorbitol to the reaction mixture resulted in the production of novel DP 3 transglycosylation products (Fig. 19).

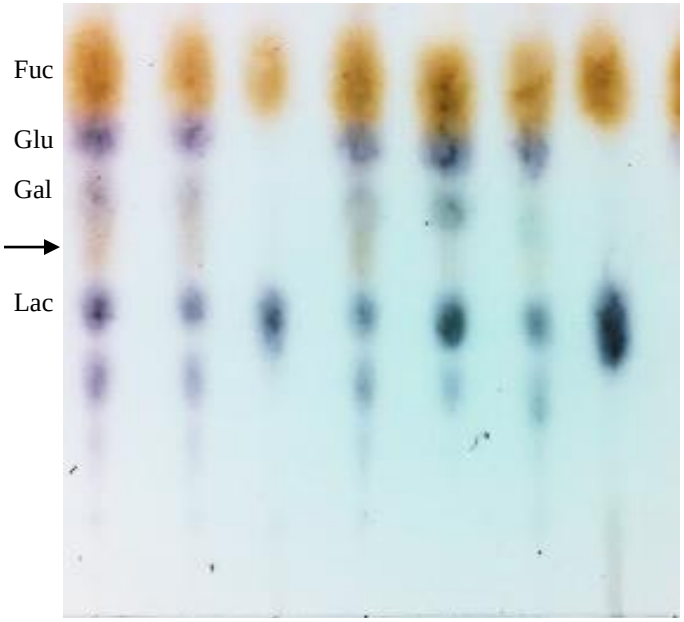

Figure 18. Effect of additional substances on transglycosylation activity of crude enzyme extracts from RD 47. C: reaction mixture with lactose and fucose.

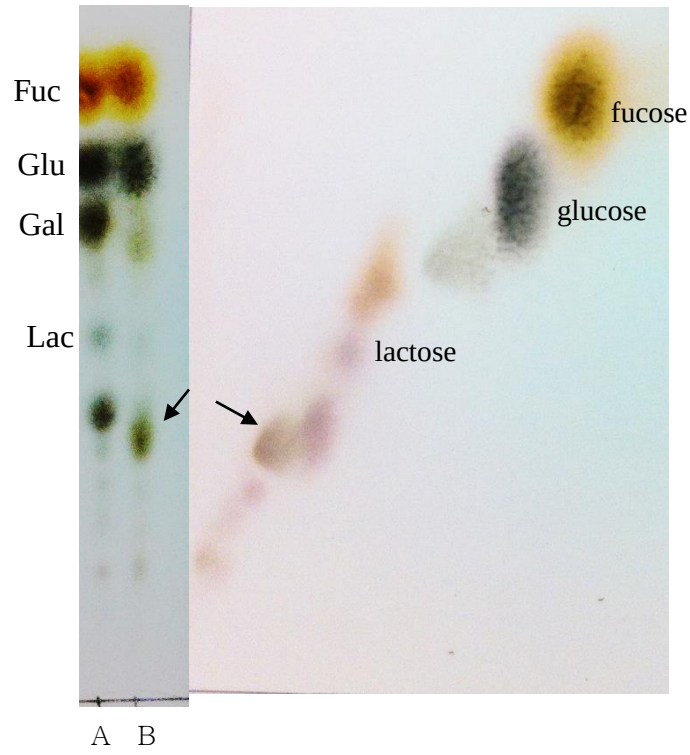

Figure 19. Transglycosylation reaction with lactose and fucose using sorbitol as an acceptor. A: reaction mixture with lactose and fucose B: reaction mixture with lactose, fucose and sorbitol.

3.3.4. Effect of *Saccharomyces cerevisiae* on oligosaccharides production

Saccharomyces cerevisiae in mixtures consumed glucose but not the other sugars (Fig. 20). However, the yield of synthesized oligosaccharides was not increased in *S. cerevisiae* treatment mixtures compared with non- treatment mixtures.

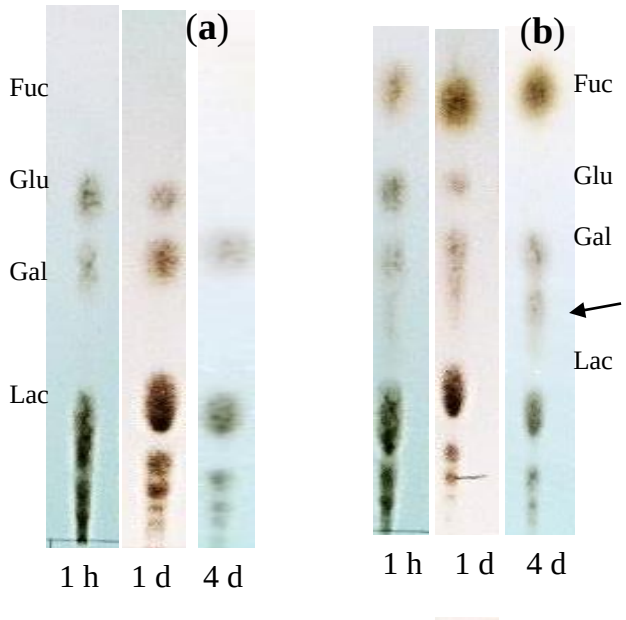

Figure 20. Transglycosylation reaction with *S. serevisiae*.

Analysis of reaction mixture with lactose (a) and lactose and fucose (b) at various temperature was performed by TLC

3.4. Characterization of the synthesized oligosaccharides

3.4.1. Mass analysis of the synthesized oligosaccharides

MALDI-TOF and LC-ESI/MS analysis suggested that the synthesized oligosaccharide comprised of fucose and glactose with DP ranging from 2 to 7. MALDI-TOF (Fig. 21 A and B) and LC- ESI/MS (Fig. 21 C and D) spectra of synthesized oligosaccharides was obtained under two different initial sugars: lactose (A and C) or lactose and fucose (B and D). Using mass spectra analysis were assessed composition of synthesized sugars and DP in the oligosaccharides. Major peaks at *m/z* 365, 527, 689, 851, 1013 and 1175 by MALDI-TOF analysis represented sodium-coordinated ($[M+Na]^+$) synthesized oligosaccharides with DP ranging from 2 to 7. The pattern of the oligosaccharides by LC-ESI/MS analysis was similar to the pattern from MALDI-TOF data.

Major peaks at *m/z* 349, 511, 673, 835 represented sodium-coordinated ($[M+Na]^+$) FUS with DP ranging from 2 to 5. LC- ESI/MS analysis allowed the detection of FUS with DP 4-7 ($[M+X]^+$ m/z 691, 848 and 1010) and DP 2 (*m/z* 344).

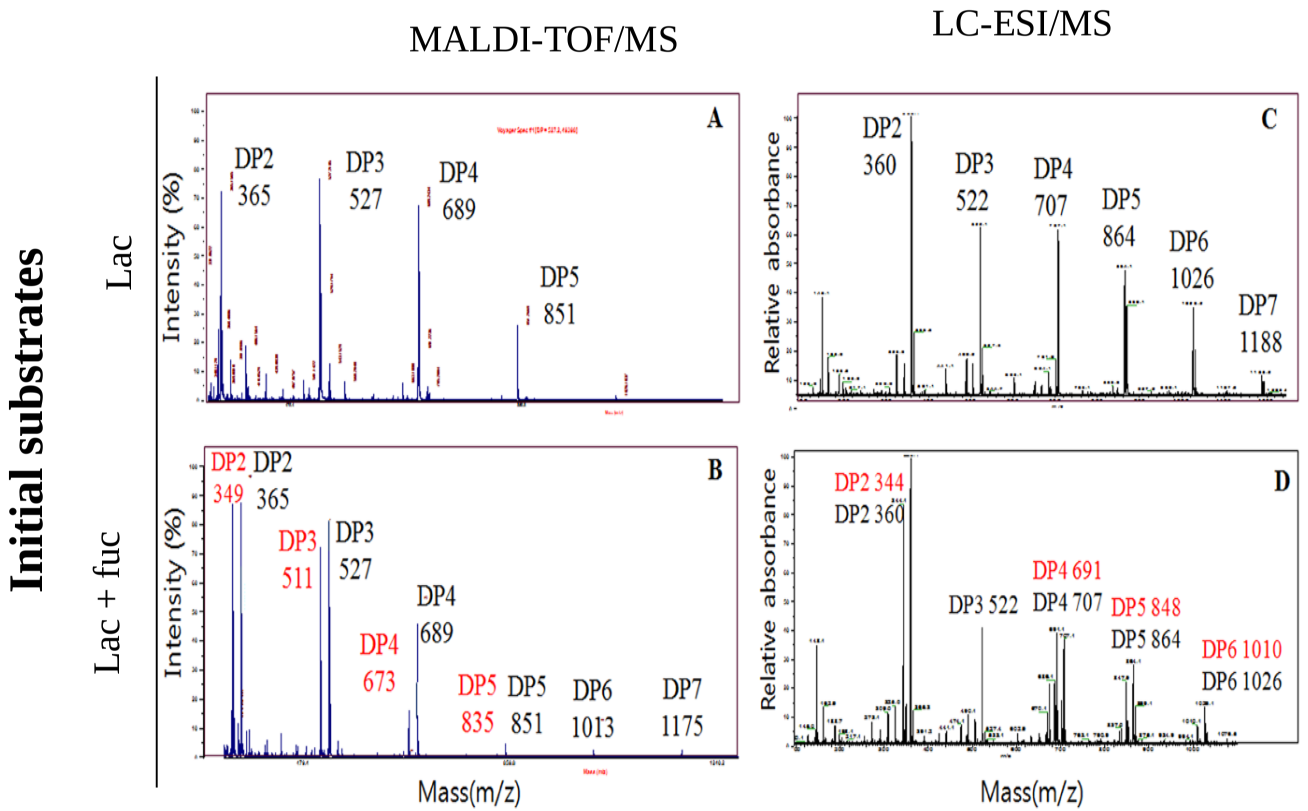

Figure 21. Mass spectra of transglycosylation products by MALDI-TOF/MS (A and B) and LC-ESI/MS (C and D) analysis. (A), (C) : Reaction mixture with lactose, (B), (D) : Reaction mixture with lactose and fucose.

3.4.2. Separation of the synthesized oligosaccharides

The results showed that synthesized oligosaccharides were separation by the size exclusion chromatography (Fig. 22). The amount of pure oligosaccharides were obtained from preparative TLC (Fig. 23 D). Preparative chromatography of fractions resulted in several polymers of different molecular weight and different sugar composition. Among them, FUS and GOS could be found.

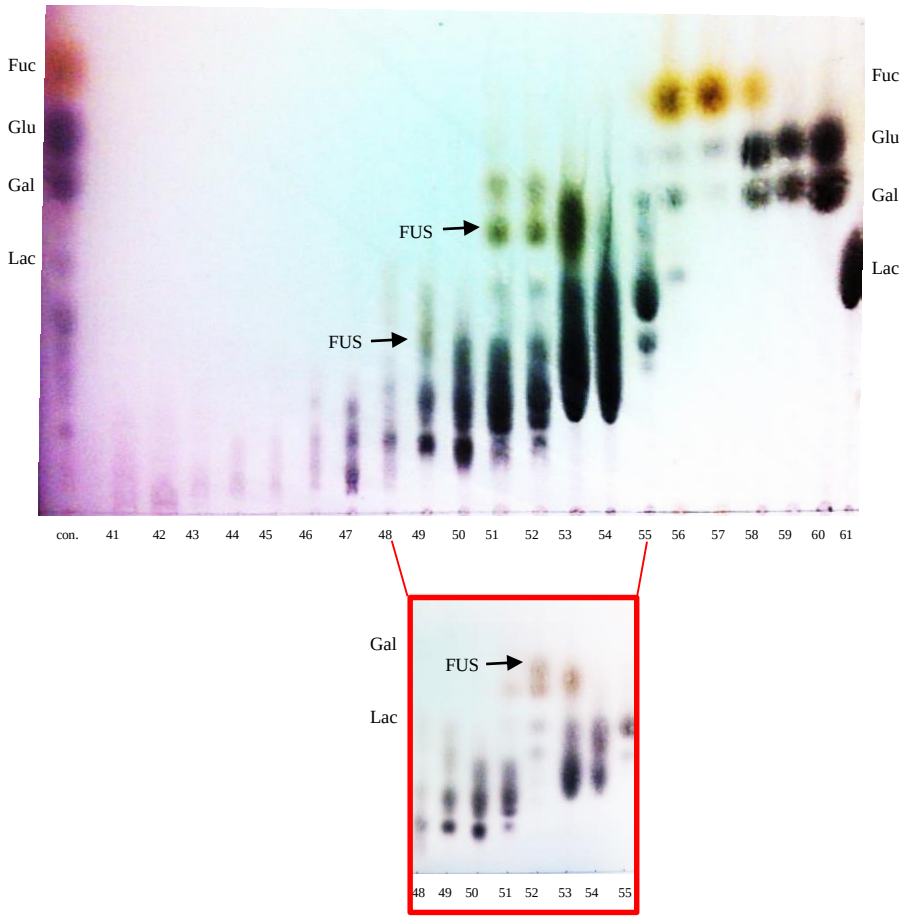

Figure 22. Separation of synthesized oligosaccharides by Bio gel P 2 column.

3.4.3. Determination of the composition of the synthesized oligosaccharides

The composition of the oligosaccharides was determined by using TLC and comparing them with standard carbohydrates (Fig. 23 A and B). Thereafter, purification of FUS was performed by using preparative TLC (Fig. 23 D). The purified FUS was degraded into L-fucose and D-galactose (Fig. 23 E) and GOS was degraded into D-glucose and D-galactose by treatment of crude enzyme extracts from RD 47 (Fig. 23 C).

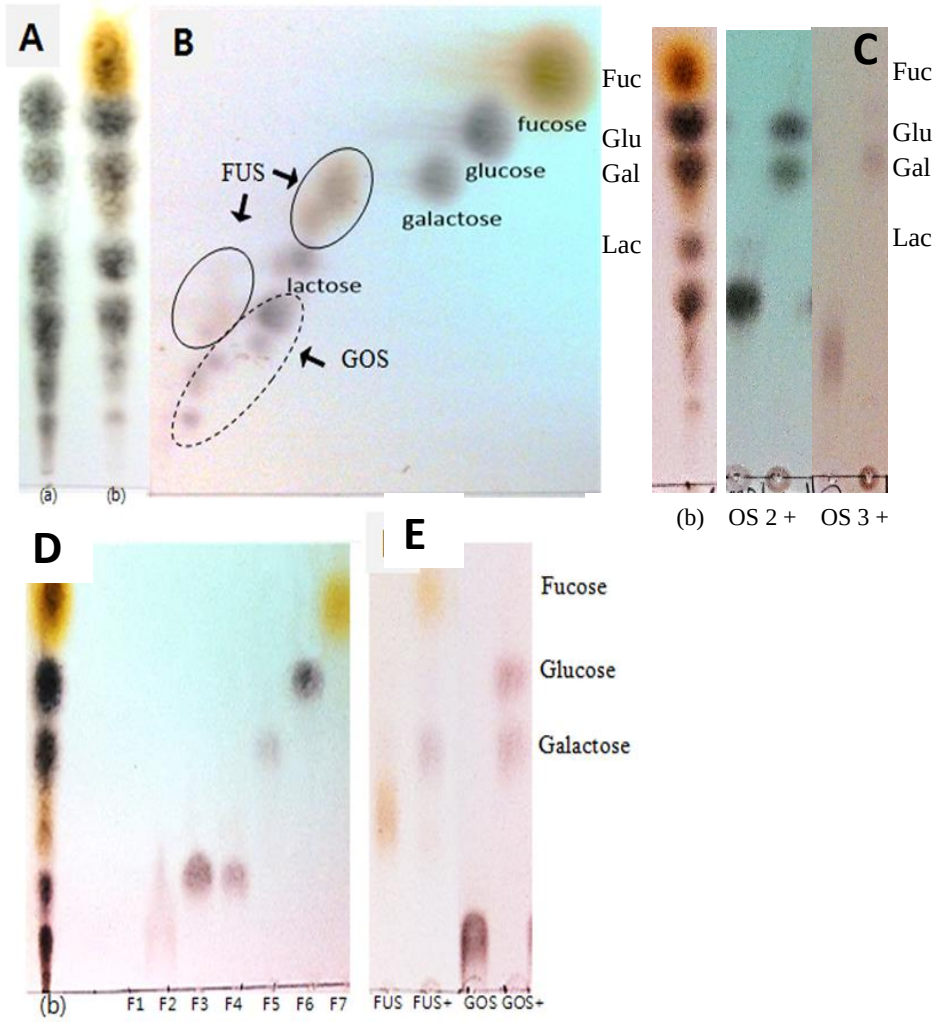

Figure 23. Determination of synthesized oligosaccharides by 1D TLC (A) and 2D TLC (B) and separation of oligosaccharides mixture (D) and hydrolysis products of synthesized sugar preparations using crude enzyme extracts from RD 47 (C, E). (A),(B),(C) were analyzed by TLC using a solvent of 1-propanol :DW: ethyl-acetate (7:2:1, v:v:v), D and E were analyzed by TLC using a solvent of 1-propanol :DW: ethyl-acetate (7:1:2, v:v:v), transglycosylation products of lactose (a) transglycosylation product of lactose and fucose (b), F 1-5 : fractioned. Oligosaccharides from (b),

4. Discussion

The aim of the present study was to assess the synthesis of a series of FUS with D-lactose and L-fucose as substrates using crude enzyme extracts from *Bifidobacteirum longum* spp. *longum* RD 47. Recent studies show continuous interest in finding industrially useful microorganisms to efficiently produce specific oligosaccharide mixture.

In the present study oligosaccharides containing both L-fucose and D-galactose were produced using crude enzyme extracts from RD 47. The results indicated that not only the acceptor L-fucose, but also the donor lactose and the hydrolytic product (galactose) of the reaction could serve as acceptors for the galactosyl moiety. The yield of oligosaccharides synthesis from lactose using glycoside hydrolases can be increased by using high substrate concentration, decreasing water activity, removing the final product and/or inhibitors from the reaction medium, and using the modifying the enzyme [22, 23].

It is crucial to find the enzyme source that provides strict selectivity, both in the position of the glycosidic linkages and in acceptors for transglycosylation. It is because different enzymes have different regiochemical specificities which affect the specificity of the glycosidic linkages and their efficiency. In general, GOS from β- galactosidases of bifidobacteria were mainly formed the β-(1-3) or β-(1-6) linkages which resulted in structurally differences from commercial GOS produced by *Kluyveromyces* spp. or *Aspergillus* spp. [24].

As shown already, the addition of raffinose in culture medium as a substitute for glucose enhanced the galactosidase activity; this supports the suggestion [25] that the induction of enzymes, which are involved in the degradation of carbohydrates, can be repressed by the presence of glucose. According to the argument, this repression of enzyme synthesis is a way of bacteria to control the oligosaccharide metabolism. When a preferred carbon source is present, there will be no unnecessary production of large amounts of enzyme. The substitution of carbohydrates for glucose in cell culture medium had a major effect on production and activity of enzyme from RD 47.

The optimal temperature for transglycosylation of the enzyme from RD 47 is almost close to its highest hydrolytic temperature, which ranged from 35 to 42℃, pH 6.3. This observation was consistent with the report [26] that the optimal condition for the transglycosylation reaction was similar to that of hydrolytic condition.

Although hydrolysis of synthesized oligosaccharides competes with transgalactosylation, the latter can be preferred at high substrate concentration, controlled temperature and lower water activity [27]. Hence, the effects of various conditions on the formation of transglycosylation products containing L-fucose unit were investigated. It has been argued that the transglycosylation of glycosidases is enhanced at a donor- acceptor ratio of 1:1 or excess of galactosyl-acceptor [28, 29]. However, the amount of oligosaccharides synthesized from lactose and fucose has decreased than that from lactose as a sole substrate, which correspond with the results from Schwab *et al*. [30]. This may be due to the fact that L-fucose has lower Michaeis constant, which is susceptible to the attack by the β-D-galactosidase than lactose or glucose [31].

Synthesized oligosaccharides were transiently formed as they were also subjected to hydrolysis, which became more accelerated toward the end of the reaction when the donor lactose was depleted. These observations were consistent with those reported for β-D-galactosidases from other microorganisms [32]. Thereafter, hydrolysis proceeded faster than synthesis, which eventually led to the accumulation of galactose and glucose.

As has been previously reported, D-fucosylglucose was synthesized using the β-D-glycosyl transferring activity of β-D-glucosidase with D-fucose and D-glucose as the D-glycosyl donor [33, 34]. In contrast to D-fucose, L-fucose used as an acceptor did not allow any formation of compounds, probably due to its inhibitory properties, while α-L-Fuc-O-Me was efficiently substituted [35].

Some of recent studies have investigated the production of FUS using *p*NP- α -L-fucoside as a fucosyl donor [36]. In an attempt to use LAB originated enzyme, the synthesis of fucosyl-N-acetylglucosamine was carried out by using α-L-fucosidase and *p*NP- α -L-fucoside partially purified from *Lactobacillus* spp. [37]. The substrate specificity for this enzyme suggests that intermediate mixtures of transient disaccharides were evident during the transfucosylation reaction. Because of these difficulties, GDP-fucose with fucosyltransferases was used to synthesize the FUS [10].

As mentioned above, using L-fucose itself as an acceptor was rarely studied, while activated forms such as Fuc-O-Me, *p*NP-fucose and GDP-fucose were often used for an L-fucose donor of transglycosylation. However, those of fucose analogs were too expensive to be used in industry.

Recently, Lee [38] reported that L-fucose was demonstrated to act as a galactosyl acceptor from *L. bulgaricus* ATCC 11842. Schwab *et al*. [29] succeeded to synthesize the FUS by *B. longum* CHCC 8700 whole cells and LAB crude cell extracts. However two previous studies reported only the formation of oligosaccharides DP 2 and DP 3 from lactose and fucose by galactosidase from LAB. Oligosaccharides with the DP ranging from 2 to 7 would be desirable prebiotics which are favorably fermented by bifidobacteria due to their structural specificity [39].

There have been few references on the formation of higher DP oligosaccharides containing L-fucose other than disaccharide. Depending on the oligosaccharide polymerization, transglycosylation products vary in terms of their health-promoting effects, as well as prebiotic activity [40]. Furthermore, tri- and tetra- oligosaccharides were not hydrolyzed *in vitro* by human digestive enzymes. A few higher-DP-oligosaccharides containing L-fucose have the ability to inhibit the adhesion or to displace adhered pathogens [41, 42]. Therefore, it is necessary to gain more insight on the formation of disaccharide, as well as the production of higher DP oligosaccharides during trans-glycosylation reaction.

Interestingly, noble transglycosylation products of DP 3 was observed from reaction with a sorbitol as substrate. Various oligosaccharide products could be obtained from multiple kinds of acceptors by the same enzymes [22].

The separation of lactose from oligosaccharides usually results in considerable losses of oligosaccharides products, mainly nonlactose-disaccharides [43, 44]. To overcome this difficulty, a process where lactose is efficiently separated from other sugars by chromatography or oxidation process was proposed by

several studies [45, 46]. Nonetheless, critical losses of oligosaccharides were unavoidable due to nonspecific separation of lactose. Still, some of the separation methods were subject to limitations of resolution and efficiency, and the combination of these methods could be used for the efficient separation of the synthesized oligosaccharides.

Furthermore, using the mixture containing lactose as a prebiotic material could have a certain benefit compared to using pure form of specific oligosaccharides. In accordance with enzymatic reaction of transglycosylation, mixtures in general contain the residual lactose, monomer sugars (glucose and galactose) and oligosaccharides of different DP [47]. After lactose was used as the initial growth source, fermentation of larger oligosaccharides was observed [48]. Therefore, in the aspect of cost and practical application, the separation of lactose from oligosaccharides would not be required.

Application of the developed FUS to the prebiotic products would be desirable for future work; most bifidobacteria could lead to various processes of FUS metabolism [49, 50]. Recently, some FUS were reported to stimulate neural connections in the brain and administration of free L-fucose in rats enhances memory retention and long-term memory potentiation (LTP) [51]. Furthermore, it would be interesting to determine the mechanism of hydrolysis and trans-galactosylation of FUS synthesizing enzymes *in vivo*. The enzymes and their products (all of which are in GRAS - generally recognized as safe - grades) presented here can be applied to various applications.

Further investigations would be needed to assess the actual effectiveness of the developed FUS compared to the real HMOs; to elucidate biochemical

properties of the individual specific oligosaccharides and characterize properties of the purified enzymes.

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국문 초록

모유 올리고당은 *Bifidobacterium* spp. 과 같은 장내 유익균주를 선택적으로 증가시키는 프리바오틱스로서 기능뿐 아니라 최근에는 유해균 및 독성물질 부착 억제, 면역 조절 작용 등을 보유하는 것으로 알려져 있다. 본 연구에서는 프로바이오틱스 균주에서 모유 올리고당의 주요 구성성분인 푸코실 올리고당을 합성하는 능력이 우수한 균주를 선발하였다. 이 균주에서 생산하는 효소를 이요하여 푸코실 올리고당의 생산을 최적화 하고자 하였다.

선발된 균주인 *B. longum* RD 47 에서 추출한 조효소와 모유 올리고당의 주요 성분인 L-푸코오스와 D-젖당을 기질로 하여 반응하였을 때 특정형태의 푸코실 올리고당 및 갈락토 올리고당이 생산됨을 확인하였다. 정성 및 질량 분석을 한 결과 합성된 올리고당의 중합도는 2~7 사이였으며 L-푸코오스를 함유한 올리고당과 갈락토오스를 함유한 올리고당의 혼합형태로 존재함을 알 수 있었다. 이는 갈락토시다아제가 기질인 젖당를 분해하는 과정에서 L-푸코오스와 분해 산물인 갈락토오스가 수여자로 작용하여 중합 반응의 결과로 사료된다.

선발된 균주의 세포 내액에서 효소 활성이 가장 높았으며 효소를 추출하는 방법으로 초음파분쇄기를 이용하였을 때 짧은 시간 내 높은 활성의 효소를 회수하는데 적합하였다. 배양 조건으로는 배지에 탄소원으로 기존 MRS 배지에 덱스트란 대신 라피노오스를 2%

첨가한 뒤 18 시간 배양액에서 추출한 효소의 활성 높았으며 올리고당 생성 반응이 가장 우수하였다. 이 균주는 탄소원에 따라 알파/ 베타 갈락토시다아제 생성이 유도되며 라피노오스에 의해 그 활성이 증가되는 특징을 지녔다. 반응조건으로는 37℃ pH 6.6 에서 합성 올리고당의 수율이 가장 높았다. 효소의 활성은 저장시간이 길어지고 온도가 높아질수록 감소하였다. EDTA 를 첨가하였을 때 온도에 의한 활성 감소가 줄어들었다.

본 연구를 통해 합성된 올리고당은 프리바이오틱스로서의 활성 외에 다양한 기능성을 보유한 것으로 사료되며 모유 올리고당을 이용한 식의약 소재, 개발의 기초 자료로 활용할 수 있다.

주요어: 푸코오스, 올리고당, 비피도박테리움, 합성

학 번: 2012-21499